JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2001, p. 3213–3221 Vol. 39, No. 9
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.9.3213–3221.2001

Use of Serum Immune Complexes in a New Test That Accurately Confirms Early Lyme Disease and Active Infection with *Borrelia burgdorferi*

MICHAEL BRUNNER[1] AND LEONARD H. SIGAL[1,2]*

Department of Medicine[1] and Departments of Pediatrics and Molecular Genetics & Microbiology,[2] UMDNJ-Robert Wood Johnson Medical School, New Brunswick, New Jersey

Received 26 March 2001/Returned for modification 2 June 2001/Accepted 23 June 2001

The present recommendation for serologic confirmation of Lyme disease (LD) calls for immunoblotting in support of positive or equivocal ELISA. *Borrelia burgdorferi* releases large quantities of proteins, suggesting that specific antibodies in serum might be trapped in immune complexes (ICs), rendering the antibodies undetectable by standard assays using unmodified serum. Production of ICs requires ongoing antigen production, so persistence of IC might be a marker of ongoing or persisting infection. We developed an immunoglobulin M (IgM) capture assay (EMIBA) measuring IC-derived IgM antibodies and tested it using three well-defined LD populations (from an academic LD referral center, a well-described Centers for Disease Control and Prevention (CDC) serum bank, and a group of erythema migrans patients from whose skin lesions *B. burgdorferi* was grown) and controls (non-Lyme arthritis inflammatory joint disease, syphilis, multiple sclerosis, and nondisease subjects from a region where LD is endemic, perhaps the most relevant comparison group of all). Previous studies demonstrated that specific antigen-antibody complexes in the sera of patients with LD could be precipitated by polyethylene glycol and could then be disrupted with maintenance of the immunoreactivity of the released antibodies, that specific anti-*B. burgdorferi* IgM was concentrated in ICs, and that occasionally IgM to specific *B. burgdorferi* antigens was found in the IC but not in unprocessed serum. EMIBA compared favorably with commercial and CDC flagellin-enhanced enzyme-linked immunosorbent assays and other assays in confirming the diagnosis of LD. EMIBA confirmed early *B. burgdorferi* infection more accurately than the comparator assays. In addition, EMIBA more accurately differentiated seropositivity in patients with active ongoing infection from seroreactivity persisting long after clinically successful antibiotic therapy; i.e., EMIBA identified seroreactivity indicating a clinical circumstance requiring antibiotic therapy. Thus, EMIBA is a promising new assay for accurate serologic confirmation of early and/or active LD.

Lyme disease is a potentially multisystem inflammatory disease caused by *Borrelia burgdorferi* (56). In the absence of erythema migrans (EM), no symptoms and signs are uniquely diagnostic of Lyme disease. Culturing *B. burgdorferi*, finding its antigens by immunohistochemistry, or identifying its DNA by PCR in biopsies are all problematic, so indirect markers are used to corroborate infection, usually measurement of antibodies by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Serum immunoglobulin M (IgM) anti-*B. burgdorferi* antibodies are often detected in early disease; by 6 to 8 weeks IgG is detected in the majority of untreated patients. Criteria for immunoblot interpretation are widely accepted (11, 16, 17), but testing is not standardized (3, 14, 26, 35, 50) and may be falsely positive due to IgM rheumatoid factor (5, 34) in other diseases (30, 37, 39, 60) and in otherwise healthy individuals (12). Clinical features of Lyme disease can develop even before the elaboration of the humoral immune response to *B. burgdorferi*. Early in the evolution of the humoral response, seronegativity might be due to the fact that specific antibodies to *B. burgdorferi* antigens are bound up in circulating immune complexes (a period of "antigen excess"), rendering the antibodies immeasurable by standard techniques (8, 48). Elevated levels of circulating immune complexes were one of the earliest described immunologic phenomena in Lyme disease (24, 25).

Overuse of testing (33, 44, 51) contributes to the misdiagnosis of Lyme disease (54, 55, 58; L. H. Sigal, Editorial, J. Infect. Dis. **171:**423–424, 1995), based on the common mistaken belief that a "positive test" is synonymous with "active infection." Persisting seropositivity may be incorrectly interpreted as ongoing infection. Seropositivity is difficult to interpret in patients with posttreatment residual or new symptoms (9, 54, 55), in whom persisting infection is a concern (1, 2, 27, 47, 55, 62). The frequency of false-positive (FP) ELISA results dictates a two-tier strategy (immunoblot confirmation of positive or equivocal ELISA [2–4]).

Other assays include indirect immunofluorescence (IFA) (36, 43), borreliacidal activity (10), and PCR (45, 58). Identification of specific immunoreactivity at the site of inflammation (e.g., antibodies in synovial or cerebrospinal fluid compared to serum) is useful but often cumbersome in identifying local infection (53, 57, 59). A simple assay is needed that can reproducibly confirm early and/or active *B. burgdorferi* infection. Attempts to improve ELISA have included antibody capture (6, 23, 32); new antigenic preparations (20, 38), including flagellin enhancement (22, 29), recombinant proteins (18, 40), or individual epitopes (28, 31, 61); and testing of antibodies contained within polyethylene glycol (PEG) precipitates of serum (13, 48, 49). Our previous studies were the first to find both *B. burgdorferi* antigens and IgM anti-

* Corresponding author. Mailing address: Division of Rheumatology MEB484, 1 Robert Wood Johnson Pl., New Brunswick, NJ 08903-0019. Phone: (732) 235-7702. Fax: (732) 235-7238. E-mail: sigallh@umdnj.edu.

bodies to these antigens within disrupted PEG precipitates from the sera of patients with Lyme disease (8). We were able to prove that the PEG precipitates contained immune complexes (ICs) by purifying the antigens only through their firm binding to serum antibodies (8). We used several of these improvements to develop an assay that, in the serum banks tested, was sufficiently sensitive to confirm early infection, sufficiently specific to obviate immunoblot confirmation, and capable of differentiating active infection from persisting seropositivity in patients with successfully treated disease. In our studies the enzyme-linked, IgM capture, IC, biotinylated antigen assay (EMIBA) fulfills these expectations.

MATERIALS AND METHODS

Growth and preparation of *B. burgdorferi* sonicate. High-passage *B. burgdorferi* strain B-31 (54) was grown in Barbour-Stoenner-Kelly medium (4, 46) (made or purchased from Sigma, St. Louis, Mo.) supplemented with 6% normal rabbit serum (Gemini Bio-Products, Calabasas, Calif.), grown in T flasks (Corning Glass, Corning, N.Y.) at 32°C. Four hundred milliliters of late-log-phase culture was harvested by centrifugation (RC5C; Sorvall-DuPont, Wilmington, Del.) at 9,000 × *g* for 15 min and was washed three times with cold phosphate-buffered saline (PBS), pH 7.2. The final pellet was resuspended in 2 ml of PBS and sonicated (Braun-Sonic 2000) medium setting for four 30 pulses with a 1-min rest between pulses. Approximately 9 mg of protein/ml was obtained, assayed with bicinchoninic acid protein (Pierce, Rockford, Ill.) and stored at −70°C until use. In certain studies a low-passage N40 (provided by Stephen Barthold, Yale University) was propagated and processed as described above (data not shown). The results shown in the paper utilize the B-31 strain, since this is the organism used in the commercial ELISA and immunoblot kits used for comparison. In side-by-side studies, results gained by using N40 and B-31 were identical.

Biotinylation of sonicate. Different long-arm biotin hydroxysuccinamide esters, including biotinamidocaproate *N*-hydroxysuccinimide (NHS) ester (Sigma) and NHS-LC-biotin II (Pierce) were equally effective; studies described in this paper used biotinamidocaproate NHS. One milliliter of a 9-mg/ml protein sonicate solution was adjusted to pH 9 by adding 0.1 ml of 0.5 M carbonate-bicarbonate buffer, pH 9, immediately before biotinylation. Optimal biotinylation was achieved using 50 mg of biotin ester/ml in dimethylformamide. To the pH-adjusted protein sonicate, 20 μl of biotin solution was added in a 16-by 100-mm glass tube, covered, and slowly rotated for 1 to 2 h at room temperature, pipetting up and down every 15 min. The reaction was stopped by adding 0.1 ml of 1 M Tris-HCl, pH 7.5. Biotinylated sonicate (Bb-bio) was dialyzed against 3 2-liter changes of PBS, pH 7.2, containing 0.02% sodium azide in the cold using a Slide-A-Lyzer (Pierce) with a 10-kDa cutoff. The resulting suspension was assayed for protein bicinchoninic acid, aliquoted, and frozen at −70°C with typical yields of at least 75%.

Serum and plasma collection. For serum, blood was drawn and allowed to clot at room temperature for 1 h; for plasma, heparinized blood was left at room temperature for 1 h. Both types of specimens were then centrifuged in a Sorvall RC5C HS-4 rotor at 769 × *g* for 10 min. Clear serum or plasma was drawn off by pipette. After serum and plasma samples were utilized in the serologic tests under study, the samples were stored at −70°C until needed for possible further testing. Freeze-thaw slightly decreased the reactivity of some samples but did not reduce any positive samples into the negative range.

Samples were obtained from three Lyme disease patient populations: (i) One hundred thirty-one came from patients evaluated at the Lyme Disease Center at Robert Wood Johnson Medical School (RWJMS) for possible Lyme disease. Before testing in our laboratory and based solely on clinical information, one author (L.H.S., who had seen all patients at the Center) designated patients as having active Lyme disease, prior Lyme disease without evidence of current active infection (prior), or no evidence of prior or present Lyme disease; results of the clinical evaluation were unknown to the laboratory personnel performing EMIBA. Patients were considered to have an active infection if they had present or previous EM or objective features of early disseminated (carditis or neurologic features, including lymphocytic meningitis, cranial nerve palsy, or radiculoneuritis) or late (arthritis or tertiary neuroborreliosis) Lyme disease and had not yet received an adequate course of antibiotic therapy for their Lyme disease (1). Patients with prior Lyme disease who had received appropriate antibiotic therapy and at the time of phlebotomy had no evidence of then-active Lyme disease of the skin, heart, joints, or peripheral or central nervous system, i.e., those who had prior Lyme disease that had been antibiotically cured, were identified as having prior infection. All samples were tested by University Diagnostics Laboratory's Lyme Disease Laboratory using IgG- or IgM-isotype-specific ELISA and immunoblot kits (MarDx, Carlsbad, Calif.) following the manufacturer's directions (1:100 dilution) and interpretation (11). In some studies IgM immunoblots using serum at 1:10 dilution were compared with ICs (1:10 dilution optimal) following the manufacturer's instructions. Collection and use of blood samples were approved by the RWJMS Institutional Review Board (Protocol W-0093). (ii) Forty-two sera within a blinded Centers for Disease Control and Prevention (CDC) collection (generously provided by Martin Schriefer, Diagnostic and Reference Section, Bacterial Zoonoses Branch, CDC, Atlanta, Ga.) were previously tested by commercial kit IgG or IgM ELISA (MarDx), CDC's flagellin-enriched IgM and IgG ELISA (46) and commercial kit IgM or IgG immunoblots (MarDx); clinical information and results of testing were supplied after completion of our studies. Based on the clinical information provided by the CDC after laboratory testing was completed, 38 patients could be assigned to active or prior groups by one author, using the above criteria (L.H.S.). (iii) Eleven sera came from patients with EM biopsy culture-proven *B. burgdorferi* infection (generously provided by Paul Mitchell, Marshfield Clinic, Marshfield, Wis.). Sera had previously been tested using IgM IFA (43), IgM enzyme immunoassay (EIA), IgM immunoblot, and polyvalent EIA, although results were withheld until completion of EMIBA studies. Sera were obtained at the time of biopsy before treatment with antibiotics—all had active Lyme disease (41, 42). Samples were obtained from three non-Lyme disease control populations: (i) Sera from 12 multiple sclerosis patients were obtained from Christine Rohowsky-Kochan, Department of Neurosciences, University of Medicine and Dentistry of New Jersey, New Jersey Medical School (Newark, N.J.). (ii) Twenty-two Venereal Disease Research Laboratory (VDRL)-positive sera (at titers between 1:2 and 1:256) from patients with syphilis were obtained from Cindy Bartlett and Marion E. Pierce, Director, Public Health and Environmental Laboratory of the New Jersey Department of Health and Senior Services, (Trenton, N.J.). No clinical information concerning these 22 patients was available for further analysis. Two blinded VDRL-negative controls were included with this serum collection. (iii) Sera from patients with inflammatory joint disease (five with systemic lupus erythematosus, eight with rheumatoid arthritis, and two with gout) were obtained from the clinical practice of one of the authors (L.H.S.).

IC precipitation. The PEG method was used to isolate ICs (15, 49). Samples (100 μl) were placed in a microfuge tube (Eppendorf) and precipitated with an equal volume of a 7% PEG (average molecular weight, 8,000; Sigma) and 0.44% NaCl in 0.1 M sodium borate buffer, pH 8.4. Tubes were vortexed, left at 4°C for at least 4 h (15), and centrifuged at 10,000 rpm (8,320 × *g*) for 15 min in the cold. Supernatants were carefully removed with a pipette. The pellet was resuspended and washed twice with 200 μl of 3.5% PEG solution in the same buffer. After the second spin, samples were resuspended in 100 μl of 0.1 M sodium borate buffer, pH 10.2; high pH buffer is better than PBS for dissociating ICs and does not affect antibody stability (P. Coyle, M. Brunner, and S. Schutzer, unpublished data). Dissociated ICs were kept in buffer at 4°C until use. There was no discernible loss of reactivity after 2 weeks of storage (M. Brunner, unpublished data).

EMIBA. One hundred microliters of affinity-purified goat anti-human IgM (mu chain specific) (KPL; Gaithersburg, Md.) per well at 10 μg/ml in 0.04 M–0.0357 M carbonate-bicarbonate coating buffer, pH 9.6, was added to Immulon 4 (Dynatech) microtiter plates. The plates were rotated slowly at room temperature for 2 h and were stored covered at 4°C overnight. The plates were warmed to room temperature and were washed three times with 10 mM PBS, pH 7.5, containing 0.1% bovine serum albumin (Sigma) and 0.05% Tween 20 (PBS-BT) using an automated plate washer (Bio-Tek ELP 35). After the final wash, 0.35 ml of blocking buffer (PBS-BT containing 5% nonfat dry milk)/well was added, and the plates were covered with Mylar. Their contents were incubated for 1 h at 37°C and were washed twice with PBS-BT. One hundred microliters of dissociated immune complexes or serum per well at 1:100 dilution in PBS-BT with 3% fish skin gelatin (Sigma) and 1% heat-inactivated normal goat serum (Vector Labs, Burlingame, Calif.) were added in duplicate. Plate contents were incubated for 2 h and washed three times with PBS-BT, and biotinylated *B. burgdorferi* was added. The plate was covered and rotated slowly for 1/2 h at room temperature.

Previous studies of IC reactivity in sera of patients with Lyme disease by others compared IC at a 1:10 dilution with unprocessed serum (representing uncomplexed antibodies and referred to in this paper as "free antibodies") at a 1:100 dilution (24, 25, 48); a 1:100 dilution of serum is used in the MarDx ELISA and immunoblot kits employed in our studies. We previously compared reactivity of ICs at a 1:10 dilution with 1:10 and 1:100 dilutions of unprocessed serum (free antibodies) and confirmed that the greater reactivity of ICs with *B. burgdorferi* proteins was not simply due to dilution of the IgM antibodies within the free-antibody fraction (8). Thus, in these studies the dilutions used were serum at 1:100 and dissociated IC at 1:10.

After 3 PBS-BT washes, a 1/8,000 dilution of peroxidase-labeled goat antibiotin (Vector Labs) in incubation buffer was added, covered, slowly rotated for 1/2

h, and washed on a plate washer for three cycles with PBS-BT, followed by two manual PBS washes. One hundred microliters of a two-component 3,3′5,5′ tetramethylbenzidine (TMB) substrate solution (KPL)/well was added. Plates were tapped lightly several times and were observed for color development, and the reaction was stopped after 10 minutes with 100 μl of 1 M H_3PO_4. Results were noted as the average optical density (OD) of duplicate samples.

EMIBA was developed so that the same positive controls in each run gave an OD of approximately 1.0. Negative controls obtained from the University Diagnostic Laboratory of RWJMS (all resided in New Jersey or surrounding areas and were without a known history of Lyme disease) gave an OD of less than 0.1. Wells coated with anti-IgM but no serum added gave an OD of 0.05 or less when read at dual wavelength (450 and 630 nm; signal at 450 nm and background at 630 nm) on a Bio-Tek EL312E ELISA plate reader. The optimal amount of Bb-bio was determined for each batch (4 to 12 μg/ml). Each batch of Bb-bio was standardized with the preceding lot using the same positive and negative sera. The new preparation was used at a concentration such that the ODs obtained from EMIBA using the new Bb-bio lot were within 5% of the ODs using the preceding batch, a procedure that we have used previously (7, 8).

The positive cutoff for each plate was the mean of 10 negative control samples run in duplicate plus 3 standard deviations (7, 8, 49, 49a). Dividing the average OD of the patient sample by the cutoff gave an index value. Index values equal to or greater than 1.0 were considered positive; less than 1.0 was negative.

Statistical analysis. Based on active or prior status of Lyme disease at the time of phlebotomy, test results were designated true positive (TP—had active LD and a positive test result), true negative (TN—did not have active LD had a negative test result), FP (did not have active LD but had a positive test result), or false negative (FN—had active LD but had a negative test result). Sensitivity and specificity were calculated as follows:

$$\text{Sensitivity} = [\text{TP}/(\text{TP} + \text{FN})] \times 100$$

$$\text{Specificity} = [\text{TN}/(\text{TN} + \text{FP})] \times 100$$

Confidence intervals of 95% for sensitivity and specificity were calculated using the Fleiss correction (19). The significance of differences in the sensitivity and specificity of the different assays tested were assessed by McNemar's chi-square test (52). Also provided are 95% confidence intervals of differences between the sensitivities and specificities of the different assays. As collections were stratified into active and prior groups, the number of samples within some cells was too small to calculate confidence intervals.

RESULTS

Comparison of EMIBA with standard serologic assays in patients with Lyme disease and non-Lyme disease controls. At the time of evaluation and phlebotomy, 64 of the 131 patients from the Lyme Disease Center were clinically designated as having active disease (diagnosed with Lyme disease but receiving no prior antibiotic treatment), 28 had prior Lyme disease (had previously undergone antibiotic treatment, with no evidence of active Lyme disease at the time of phlebotomy), and 39 had never had Lyme disease. The sensitivity and specificity of each assay were calculated (Table 1). We then determined if the sensitivity and specificity of the EMIBA and the free-antibody assay were superior to the sensitivity and specificity of each of the comparator assays and if these differences were statistically significant (Table 2).

EMIBA results correlated better with clinical findings than did the free-antibody assay, although the difference was not statistically significant (Table 1). Both EMIBA and the free-antibody assay were significantly more sensitive than all other assays ($P < 0.001$); for each comparison with the other assays, EMIBA was slightly superior to the free-antibody assay (Table 2).

EMIBA was slightly more specific than the free-antibody assay (Table 1), and both were more specific than the other assays (Table 2). Some of these differences reached statistical significance: EMIBA was more specific than the IgM ELISA ($P < 0.002$) and IgM immunoblot ($P = 0.022$), while the free-antibody assay was superior only to the IgM ELISA ($P = 0.012$). The IgG immunoblot was as specific as either experimental assay.

TABLE 1. Results of EMIBA, free-antibody assay, IgM- and IgG-isotype-specific ELISA, and IgM- and IgG-isotype-specific immunoblotting[a]

Assay type	Sensitivity (%)	95% CI for sensitivity (%)	Specificity (%)	95% CI for specificity (%)
EMIBA[b]	98	90–100	96	87–99
Free antibody[c]	95	86–99	91	81–96
IgM ELISA[d]	66	53–77	76	64–86
IgG ELISA	58	45–70	87	76–93
IgM immunoblotting[d]	58	45–70	84	72–91
IgG immunoblotting	44	32–57	93	83–97

[a] Patients ($n = 131$) were evaluated at the Lyme Disease Center for possible Lyme disease. Of the 131 patients, 64 had active Lyme disease at the time of phlebotomy, 28 had evidence of prior Lyme disease but not of active infection at the time of phlebotomy, and 39 had no evidence of present or past Lyme disease. See Appendix, Table A1, for raw data. CI, confidence interval.
[b] Assay utilizes antibodies derived from ICs prepared from serum.
[c] Assay system is same as that of EMIBA but utilizes serum not processed for ICs.
[d] Immunoblotting and ELISA are MarDx assays.

After unblinding the CDC serum collection, we calculated sensitivity and specificity in the 38 samples that could be designated as being from active or prior patients (Table 3). EMIBA and the free-antibody assay were 100% sensitive, somewhat better than all but the CDC assay; these differences were not statistically significant.

The EMIBA had a higher specificity than the free-antibody assay, a difference significant at $P < 0.05$. The sensitivities of both experimental assays were significantly superior to that of the flagellin-enhanced CDC ELISA ($P < 0.001$ for both comparisons) (Table 3). Thus, in a blinded, independent, well-defined serum collection, EMIBA and, to a lesser degree, the free-antibody assay outperformed the other assays. For all assays specificities were lower in analysis of the CDC serum collection than in studies of sera obtained locally (Table 1).

EMIBA detection of serologic reactivity in EM (early Lyme disease) compared with serodetection utilizing standard assays. Twenty-seven of 131 specimens from the Lyme Disease Center were from patients with EM, 24 before treatment. The other three had been successfully antibiotically treated 3 to 9 months before phlebotomy; none had evidence of infection at the time of phlebotomy. These three constitute the TN and FP groups in Table 4 (see Appendix). EMIBA and the free-antibody assay were more sensitive than comparison assays and more specific than all but the IgG ELISA, to which they were equivalent. Thus, EMIBA and the free-antibody assay were superior to other assays in corroborating early Lyme disease.

Within the CDC panel were 28 samples from EM patients (Table 5). EMIBA, the free-antibody assay, the IgM ELISA, and the flagellin-enhanced CDC ELISA were 100% sensitive, with IgG ELISA the least sensitive assay. The specificities of EMIBA, the IgG ELISA, and the IgG immunoblot assay were comparable, but the specificity of the CDC ELISA was substantially lower.

In sera from 11 patients with culture-positive EM from the Marshfield Clinic, EMIBA was positive in eight, the free-antibody assay in seven, the IgM IFA in seven, the IgM EIA in

TABLE 2. Differences between EMIBA[a] or free-antibody assay[b] and standard assays in terms of sensitivity and specificity for Lyme disease Center samples

Assay type	Difference in sensitivity (%)		95% CI[e] for sensitivity (%)		Difference in specificity (%)		95% CI for specificity (%)	
	EMIBA	Free antibody	EMIBA	Free antibody	EMIBA	Free antibody	EMIBA	Free antibody
Free antibody	NS[d]	—			NS	—		
IgM ELISA[c]	33	30	20–46	17–43	19	15	6–32	4–26
IgG ELISA	40	38	27–53	25–51	NS	NS		
IgM immunoblotting[c]	40	38	27–53	25–51	12	NS	3–21	
IgG immunoblotting	55	52	40–70	39–65	NS	NS		

[a] Assay uses antibodies derived from ICs prepared from serum.
[b] Assay system is same as that of EMIBA but utilizes serum not processed for ICs.
[c] ELISA and immunoblotting are MarDx assays.
[d] NS, no statistically significant difference.
[e] CI, confidence interval. We evaluated the 95% CI of differences between EMIBA or the free antibody assay and the other assays in term of specificity and sensitivity.
[f] —, no comparison of free-antibody sensitivity and specificity with itself.

three, the IgM immunoblot assay in four, and the polyvalent EIA in three. EMIBA was positive in three IFA-negative samples; the IFA was positive in two EMIBA-negative specimens. The free-antibody assay did not detect seroreactivity in any EMIBA-or IFA-negative sera. In only one of the samples were both EMIBA and the free-antibody assay negative. Thus, in a small number of culture-proven EM patients, EMIBA was superior to other assays, although only marginally better than the free antibody assay or the IFA.

EMIBA is able to differentiate between seropositivity in patients with ongoing infection and persisting seropositivity related to past infection (active versus prior disease). Of 131 Lyme Disease Center patients, 64 had active infection and 28 had previous Lyme disease without evidence of active disease at the time of phlebotomy, i.e., previously cured Lyme disease (Table 6). Table 6 presents serologic results in the comparator assays with reference to results in EMIBA and the free-antibody assay: (i) free-antibody assay (+), EMIBA (−); (ii) free-antibody assay (−), EMIBA (+); (iii) free-antibody assay (+), EMIBA (+); and (iv) free-antibody assay (−), EMIBA (−).

EMIBA was positive in 62 of 64 samples of patients with active Lyme disease, and the free-antibody assay was positive in 63. The other assays were less often positive in patients with active and ongoing infection. Only the IgM ELISA was positive in nearly two-thirds of active patients, while the IgG immunoblot assay was positive in only 44%. In the two EMIBA-negative samples, neither isotype-specific immunoblot assay was positive.

In 24 of 28 prior patients, EMIBA was negative; the free-antibody assay was negative in 26. In these patients without active infection, between 5 and 12 samples were positive in each of the other assays. Thus, EMIBA and the free-antibody assay performed better in samples from patients with active and prior infection than the other assays.

Of the 38 assignable CDC samples, 9 were designated as active infection and 29 were designated as prior Lyme disease. The results in Table 7 are presented in the same manner as in Table 6. All nine of the CDC samples from patients with active infection were positive by EMIBA and the free-antibody assay (Table 7); of the other assays, only the CDC ELISA was positive in all patients. In patients with prior disease, the assay most often negative would be the best at differentiating prior from active disease (a positive result in a person with infection that is no longer active is, in essence, an FP). Fourteen of the

TABLE 3. Results of EMIBA, free-antibody assay, IgM- and IgG-isotype-specific ELISA, IgM- and IgG-isotype-specific immunoblotting, and CDC flagellin-enhanced polyvalent ELISA for CDC serum samples[i]

Assay type	Sensitivity (%)	95% CI[e] for sensitivity (%)	Specificity (%)	95% CI for specificity (%)
EMIBA[a]	100	63–99	66[f,g]	46–81
Free antibody[b]	100	63–99	48[h]	30–66
IgM ELISA[c]	78	40–96	43	25–63
IgG ELISA	78	40–96	57	39–75
IgM immunoblotting[c]	56	23–85	62	42–79
IgG immunoblotting	89	51–99	59	39–76
CDC ELISA[d]	100	63–99	17	7–36

[a] Assay utilizes antibodies derived from ICs prepared from serum.
[b] Assay system is same as that of EMIBA but utilizes serum not processed for ICs.
[c] Immunoblotting assay and non-CDC ELISA are commercially available MarDx kits.
[d] Flagellin-enhanced ELISA was developed by the CDC and was reported previously (29).
[e] CI, confidence interval.
[f] The difference between the specificity of EMIBA and that of the free-antibody assay was 17% (95% CI, 0 to 34%; $P < 0.05$).
[g] The difference between the specificity of EMIBA and that of the CDC ELISA was 48% (95% CI, 25 to 71%; $P < 0.001$).
[h] The difference between the specificity of the free-antibody assay and that of the CDC ELISA was 31% (95% CI, 8 to 54%; $P < 0.001$).
[i] Results are for 38 serum samples from the CDC data bank. In the four remaining samples, clinical information was insufficient to determine if the patients had active or prior infection. See Appendix, Table A2, for raw data.

TABLE 4. Results of EMIBA, free-antibody assay, IgM- and IgG-isotype-specific ELISA, and IgM- and IgG-isotype-specific immunoblotting for 27 patients with EM[a]

Assay type	Sensitivity (%)	Specificity (%)
EMIBA[b]	100	100
Free antibody[c]	96	100
IgM ELISA[d]	58	67
IgG ELISA	54	100
IgM immunoblotting[d]	58	33
IgG immunoblotting	42	67

[a] Twenty-seven patients with EM were evaluated at the Lyme Disease Center. In 24 of the patients, EM was present at the time of phlebotomy and/or phlebotomy occurred before antibiotic treatment; the remaining three patients had received adequate antibiotic treatment and had no evidence of ongoing infection with *B. burgdorferi*. See Appendix, Table A3, for raw data.
[b] Assay utilizes antibodies derived from ICs prepared from serum.
[c] Same assay system as that of EMIBA but utilizes serum not processed for ICs.
[d] Commercially available MarDx kits.

TABLE 5. Results of EMIBA, free-antibody assay, IgM-and IgG-isotype-specific ELISA, IgM- and IgG-isotype-specific immunoblotting, and flagellin-enhanced CDC ELISA for 28 patients with EM[a]

Assay type	Sensitivity (%)	Specificity (%)
EMIBA[b]	100	73
Free antibody[c]	100	55
IgM ELISA[d]	100	33
IgG ELISA	67	71
IgM immunoblotting[d]	83	50
IgG immunoblotting	83	77
CDC ELISA	100	23

[a] Serum samples from these 28 patients were included within the CDC serum bank. See Appendix, Table A4, for raw data.
[b] Assay utilizes antibodies derived from ICs prepared from serum.
[c] Same assay system as that of EMIBA but utilizes serum not processed for ICs.
[d] Commercially available MarDx kits.

29 samples from patients with prior disease were negative by EMIBA. Nineteen of the free-antibody assay results were negative. The IgM immunoblot assay was negative in 18 samples, the IgG ELISA and the immunoblot in 17 each, the IgM ELISA in 13, and the CDC ELISA in only 5.

Thus, the free-antibody assay agreed with clinical status better than the other assays (28 of 38; 9 of 9 TPs and 19 of 29 TNs); the CDC ELISA was least likely to predict clinical status (14 of 38; 9 of 9 TPs and only 5 of 29 TNs). The other assays were not remarkably different in their agreement with clinical status: IgG immunoblot assay (correct in 25; 8 of 9 and 17 of 29); EMIBA (23; 9 of 9 and 14 of 29) and IgM immunoblot assay (23; 5 of 9 and 18 of 29); IgG ELISA (23; 7 of 9 and 16 of 29); and IgM ELISA (19; 7 of 9 and 12 of 29).

Eleven of 29 prior patients had been antibiotically treated 3 months or less prior to phlebotomy; 8 of these 11 samples were EMIBA FP results. The persistence of ICs in these samples may represent ongoing IC formation during recently active infection such that all IC had not yet been cleared from the circulation.

As noted above, 8 of 11 culture-proven EM samples EMIBA were positive; in six of these eight the free-antibody assay was also positive.

EMIBA results in control subjects: multiple sclerosis, syphilis, and rheumatic diseases. One (8%) of the 12 multiple sclerosis patients' sera tested was positive for both free antibodies and IC by EMIBA; the sample was negative by IgM immunoblotting.

In the free-antibody assay, 54.5% (12 of 22) of VDRL-positive samples were reactive, whereas 31.8% (7 of 22) were positive in EMIBA. All EMIBA-positive samples were also free antibody positive, i.e., the EMIBA positives were a subset of the free-antibody-positive group. Of the 12 free-antibody-positive sera, 6 were positive by standard *B. burgdorferi* IgM immunoblotting. Six of the 7 EMIBA-positive samples were positive by standard IgM immunoblotting. All 5 samples positive in the free-antibody assay but negative in EMIBA were immunoblot negative. Both VDRL-negative controls were negative in both EMIBA and the free-antibody assay. Thus, EMIBA was superior to the free-antibody assay (was positive in fewer syphilis sera) and in six of seven of EMIBA-positive samples detected IgM cross-reacting with *B. burgdorferi* proteins with sufficient affinity to produce a positive anti-*B. burgdorferi* immunoblot.

Of the 15 patients with active inflammatory rheumatologic disease who were tested, none was positive in either the free-antibody assay or EMIBA.

DISCUSSION

Although the diagnosis of Lyme disease should be based solely on appropriate historical and objective clinical findings, serologic evidence favoring the diagnosis may be useful or necessary in some circumstances. In the presence of EM, serologic confirmation is not necessary. However, lacking an EM lesion, an isolated virus-like syndrome following a tick bite cannot be attributed to early *B. burgdorferi* infection without laboratory confirmation. The early features of Lyme disease may occur too soon after tick bite for specific humoral immune responses to be measurable by current immunoassays. Only with highly sensitive and specific serologic or microbiological tests can unusual or atypical clinical features of *B. burgdorferi* infection be correctly included within the spectrum of Lyme disease and can illusory associations be identified as such and excluded.

Serologic tests are commonly misused as if they were "diagnostic." We favor the term "anti-*B. burgdorferi* antibody test" as preferable to the more euphonious but also more prejudicial term "Lyme disease test." Even in circumstances where the clinical likelihood of Lyme disease is very high, this can, at best, be a "seroconfirmatory," not a "serodiagnostic" test. Many serologic assays are currently available to detect anti-*B. burgdorferi* antibodies, but all share two limitations: (i) in early disease the tests may not detect low levels of specific antibody, and (ii) none differen-

TABLE 6. Results of EMIBA and free-antibody assays in 64 patients evaluated at the Lyme Disease Center who had active infection at the time of phlebotomy and in 28 patients who had a history of prior Lyme disease but no objective evidence of ongoing infection (prior) at the time of phlebotomy

Results for patients with active disease							Results for patients with prior disease						
No. of patients (total, 64)	Free[a]	EMIBA[b]	ELISA[c]		Immunoblot[c]		No. of patients (total, 28)	Free	EMIBA	ELISA		Immunoblot	
			IgM	IgG	IgM	IgG				IgM	IgG	IgM	IgG
2	+	−	1	1	0	0	1	+	−	1	1	1	1
1	−	+	0	0	1	1	3	−	+	3	1	2	1
61	+	+	41	36	36	27	1	+	+	0	0	0	0
0	−	−	0	0	0	0	23	−	−	8	3	8	3

[a] Same assay system as that of EMIBA but utilizes serum not processed for ICs. +, antibodies detected (positive test); −, no antibodies detected (negative test).
[b] Assay utilizes antibodies derived from ICs prepared from serum. +, antibodies detected (positive test); −, no antibodies detected (negative test).
[c] Commercially available MarDx kits.

TABLE 7. Results of EMIBA and free-antibody assays in nine patients included within the CDC serum bank who had active infection at the time of phlebotomy (as determined by review of the clinical information evaluated after the assays were performed) and in 29 patients who had a history of prior Lyme disease but no objective evidence of ongoing infection at the time of phlebotomy

Results for patients with active disease								Results for patients with prior disease							
No. of patients (total, 9)	Free antibody[a]	EMIBA[b]	ELISA[c]		Immunoblot[c]		CDC ELISA	No. of patients (total 29)	Free antibody[a]	EMIBA	ELISA		Immunoblot		CDC ELISA
			IgM	IgG	IgM	IgG					IgM	IgG	IgM	IgG	
0	+	−	0	0	0	0	0	0	+	−	0	0	0	0	0
0	−	+	0	0	0	0	0	5	−	+	2	3	1	3	5
9	+	+	7	7	5	8	9	10	+	+	8	5	6	6	9
0	−	−	0	0	0	0	0	14[d]	−	−	6	4	4	3	10

[a] Same assay system as EMIBA but utilizes serum not processed for ICs. +, antibodies detected (positive test); −, no antibodies detected (negative test).
[b] Assay utilizing antibodies derived from ICs prepared from serum. +, antibodies detected (positive test); −, no antibodies detected (negative test).
[c] Commercially available MarDx kits. +, antibodies detected (positive test); −, no antibodies detected (negative test).
[d] One of the 14 samples was not tested by isotype-specific ELISA.

tiates persistent seropositivity in active infection from clinically irrelevant persisting antibodies, i.e., seropositivity without evidence of active infection. We sought to develop an assay capable of satisfying both needs. We started with an IgM capture format (6, 23, 32), which diminishes high background levels and possible confounding serum components, e.g., competing IgG. We knew that the sera of many Lyme disease patients contained IgM antibodies within materials obtained by PEG precipitation, thought to be IC, and that the IgM bound within the precipitate might be undetectable in assays using unprocessed serum (13, 48, 49). Use of the IgM obtained from disrupted PEG precipitates enhanced the sensitivity of immunoblot in Lyme disease (49); these studies all used PEG precipitation, an established method of purifying IC (15), at a 1:10 dilution. Our previous studies demonstrated that this procedure did not merely concentrate serum IgM in a nonspecific fashion (8). These were the first studies to prove that the PEG precipitate actually satisfied the definition of an IC: the PEG precipitates contained antibodies specific for the *B. burgdorferi* antigen OspA, and the OspA protein was isolated from the putative IC only by its interaction with antibody within the PEG precipitate (8). The rigorous method used to prepare the material subjected to Western blot analysis assured that the OspA was within IC and not nonspecifically PEG precipitated in a high-molecular-weight complex other than a specific IC. In addition PEG precipitates contained IgM against *B. burgdorferi* proteins, in some cases antigens not recognized by the free antibodies in untreated serum (8), in agreement with previous, small studies (48, 49).

The present studies represent the largest and broadest application of IC technology to the seroconfirmation of Lyme disease and the only comparison of IC- with free-antibody-based assays and currently available immunoassays (IFA, ELISA, flagellin-enhanced ELISA, and Western blotting). Previous studies have shown that anti-*B. burgdorferi* IgM may persist in the sera of patients with Lyme disease after treatment and apparent cure (1, 27). In order to detect even very low IgM levels in later disease, we made use of the increased sensitivity intrinsic to EMIBA; we were able to further enhance sensitivity by biotinylating the sonicate, giving the same amplification benefits as in biotin-enhanced immunoblotting (49).

Lyme disease sera in this study were from three sources, and in all cases the testing was done blinded to clinical information. The first group of sera was from patients evaluated for Lyme disease at our regional referral center. This population is the most relevant to a serologic trial—patients from an area of endemicity whose symptoms and signs prompted the patients and/or their physicians to consider Lyme disease. The second group was from the CDC collection, an established serum bank used in previous serologic studies (29). The third group, from the Marshfield Clinic, represents an incontrovertible "gold standard" for early Lyme disease-culture-positive EM patients, with healthy controls interspersed.

Studies using all three serum banks showed that EMIBA can serologically confirm early Lyme disease. EMIBA and, to a lesser degree, the free-antibody assay were superior to the comparator assays. In the Lyme Disease Center sera, EMIBA was better able to differentiate between persisting seropositivity indicating active infection (thereby warranting antibiotic therapy) and seropositivity of no clinical significance, relating to prior cured infection; as noted, seropositivity can persist long after clinical cure (1). EMIBA was somewhat less effective in the CDC sera. This was most apparent in all the assays' ability to differentiate active from prior disease. We were able to assign disease status in only 38 CDC samples; clinical information was less detailed than available for the Lyme Disease Center population, especially concerning the timing of previous treatment and time between treatment and phlebotomy. Differences in EMIBA's correlation with disease status may relate to improper assignment of patient samples to active or prior groups based on incomplete information and/or the fact that a short time had elapsed from successful treatment to phlebotomy. In the latter case, FP EMIBA results might be due to the persistence of IC following treatment, a phenomenon noted subsequently in unblinded samples tested in our laboratory (M. Brunner and L. H. Sigal, unpublished observations). It is also possible that the smaller size of the CDC bank may have obscured differences in the assay's predictive power.

Sera from patients with syphilis contain antibodies that also bind to *B. burgdorferi*. In this control group EMIBA was superior to the free-antibody assay, in that fewer of the luetic sera were positive. FP results were present in other assays, as well—six of the seven EMIBA-positive samples were positive in IgM immunoblotting. A small proportion of multiple sclerosis patients also had antibodies reacting with *B. burgdorferi* proteins. We are currently exploring ways to improve the assay to minimize FP tests, especially in these groups (7).

Our results suggest that EMIBA is slightly superior to our free-antibody assay, superior to the standard assays in seroconfirmation of Lyme disease, effective in detecting antibod-

ies in early disease, better than standard assays and comparable to IFA in the small group of sera from patients with positive skin biopsy cultures, and helpful in determining the clinical significance of seropositivity. The concentration of anti-*B. burgdorferi* antibodies within IC has been demonstrated by comparative immunoblotting (8).

Future studies will include sequential samples before, during, and after antibiotic treatment of Lyme disease and sera from patients with other nonluetic spirochetal infections and from larger numbers of subjects with other rheumatologic and neurologic diseases. EMIBA utilizes IgM from IC; assays measuring the IgA and IgG within IC might also be helpful in the seroconfirmation of active infection with *B. burgdorferi*. Finally, differently grown organisms may provide a more advantageous antigen pool for testing clinically relevant seroreactivity (21). We are currently working on a quantitative method of comparing levels of free antibodies with levels of antibody within IC to improve the clinical predictive value (i.e., active versus previously cured infection) of our assay. If our current observations are borne out, EMIBA may be able to replace the current two-tiered seroconfirmation approach in Lyme disease with a single assay.

Thus, IC-based serologic assays are valuable in seroconfirmation of the diagnosis and clinical status of *B. burgdorferi* infection and might be useful in other diseases as well. In early disease, a state of antigen excess, the IgM of the humoral response to a new pathogen may be sequestered in IC, bound to circulating pathogen- or transformed cell-derived antigen targets and undetectable by standard serum-based assays. In such circumstances free-antibody levels would be undetectable. The ongoing production of ICs is dependent on and limited by the ongoing production of pathogen- or transformed-cell-derived antigens, so IC-based assays might be useful in determining if persistent seropositivity is a marker of disease persistence following treatment for infectious or malignant diseases. Our results suggest that further exploration of IC-based seroconfirmatory assays is warranted.

ACKNOWLEDGMENTS

Our thanks go to Stanley Stein (Center for Advanced Biotechnology and Medicine, UMDNJ-RWJMS and Rutgers University, Piscataway, N.J.) for reviewing the manuscript and for his many insightful comments in the development of the EMIBA technique; Paul Mitchell (Marshfield Clinic, Marshfield, WS.) for providing valuable serum samples and many valuable comments concerning this work; Martin Schriefer Bacterial Zoonoses Branch, CDC, Atlanta, Ga., Christine Rohowsky-Kochan, (Department of Neurosciences, UMDNJ-New Jersey Medical School, Newark, N.J.), and Cindy Bartlett and Marion E. Pierce (Public Health and Environmental Laboratory of the New Jersey Department of Health and Senior Services, Trenton, N.J.) for providing valuable serum samples; Martin Feuerman biostatistician, Winthrop-University Hospital, Mineola, N.Y., for many productive conversations about and help with statistical analysis; Ann Lee, doctoral student in the Rutgers University School of Education for her help in preparing figures; the patients seen at the Lyme Disease Center for their many contributions to our research and the education of trainees at UMDNJ-RWJMS; and the staff of the Lyme Disease Center and the Division of Rheumatology (Sondra Patella, Carol Squindo, Pat Hanas, Cheryl Guerard, Brenda Taylor, and Betsy Schott) for their many efforts on behalf of our patients and our research.

APPENDIX

Tables A1, A2, A3, and A4 show data that were used to calculate the sensitivity and specificity values shown in Tables 1, 3, 4, and 5, respectively.

TABLE A1. Number of positive responses in specific assay for Lyme Disease Center samples

Test result	EMIBA[a]	Free antibody[b]	ELISA[c]		Immunoblot[c]	
			IgM	IgG	IgM	IgG
TP	63	61	42	37	37	28
TN	64	61	51	58	56	62
FP	3	6	16	9	11	5
FN	1	3	22	27	27	36

[a] Assay utilizes antibodies derived from IC prepared from serum.
[b] Same assay system as EMIBA but utilizes serum not processed for IO.
[c] MarDx assay.

TABLE A2. Number of positive or negative responses in specific assay for CDC serum samples

Test result	EMIBA[a]	Free antibody[b]	ELISA[c]		Immuno-blot[c]		CDC ELISA[d]
			IgM	IgG	IgM	IgG	
TP	9	9	7	7	5	8	9
TN	19	14	12	16	18	17	5
FP	10	15	16	12	11	12	24
FN	0	0	2	2	4	1	0

[a] Assay utilizes antibodies derived from IC prepared from serum.
[b] Same assay system as EMIBA but utilizes serum not processed for IC.
[c] MarDx assay.
[d] Flagellin-enhanced ELISA. It was developed by the CDC and previously reported (29).

TABLE A3. Number of positive or negative responses in specific assay for EM patients from the Lyme Disease Center samples

Test result	EMIBA[a]	Free antibody[b]	ELISA[c]		Immunoblot[c]	
			IgM	IgG	IgM	IgG
TP	24	23	14	13	14	10
TN	3	3	2	3	1	2
FP	0	0	1	0	2	1
FN	0	1	10	11	10	14

[a] Assay utilizes antibodies derived from IC prepared from serum.
[b] Same assay system as EMIBA but utilizes serum not processed for IC.
[c] MarDx assay.

TABLE A4. Number of positive or negative responses in specific assay fo EM patients from the CDC serum samples

Test result	EMIBA[a]	Free antibody[b]	ELISA[c]		Immuno-blot[c]		CDC ELISA
			IgM	IgG	IgM	IgG	
TP	6	6	6	4	5	5	6
TN	16	12	7	15	11	17	5
FP	6	10	14	6	11	5	17
FN	0	0	0	2	1	1	0

[a] Assay utilizes antibodies derived from IC prepared from serum.
[b] Same assay system as EMIBA but utilizes serum not processed for IC.
[c] MarDx assay.

REFERENCES

1. **Aguero-Rosenfeld, M., J. Nowakowski, S. Bittker, D. Cooper, R. B. Nadelman, and G. P. Wormser.** 1996. Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. J. Clin. Microbiol. **34:**1–9.
2. **Baig, S., T. Olsson, K. Hansen, and H. Link.** 1991. Anti-*Borrelia burgdorferi*

antibody response over the course of Lyme neuroborreliosis. Infect. Immun. **59:**1050–1056.
3. **Bakken, L., K. L. Case, S. M. Callister, N. J Bourdeau, and R. F, Schell.** 1992. Performance of 45 laboratories participating in a proficiency testing program for Lyme disease serology. JAMA **268:**891–895.
4. **Barbour, A.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
5. **Barka, N., M. S. Agopian, and J. B. Peter.** 1990. False-positive IgM antibodies to Borrelia burgdorferi in indirect ELISA as a result of IgM rheumatoid factor. J. Infect. Dis. **161:**1312.
6. **Berardi, V., K. E. Weeks, and A. C. Steere.** 1988. Serodiagnosis of early Lyme disease: analysis of IgM and IgG antibody responses by using an antibody-capture enzyme immunoassay. J. Infect. Dis. **158:**754–760.
7. **Brunner, M., S. Stein, P. D. Mitchell, and L. H. Sigal.** 1998. IgM capture assay for the serologic confirmation of early Lyme disease: analyzing immune complexes with biotinylated *Borrelia burgdorferi* sonicate enhanced with flagellin peptide epitope. J. Clin. Microbiol. **36:**1074–1080.
8. **Brunner, M, and L. H. Sigal.** 2000. Immune complexes from Lyme diseae sera contain Borrelia burgdorferi antigen and antigen-specific antibodies: potential use for improved testing. J. Infect. Dis. **182:**534–539.
9. **Bujak, D., A. Weinstein, and R. L. Dornbush.** 1996. Clinical and neurocognitive features of the post Lyme disease syndrome. J. Rheumatol. **23:**1392–1397.
10. **Callister, S. M., R. F. Schell, K. L. Case, S. D. Lovrich, and S. P. Day.** 1993. Characterization of the borreliacidal antibody response to Borrelia burgdorferi in humans: a serodiagnostic test. J. Infect. Dis. **167:**158–164.
11. **Centers for Disease Control and Prevention.** 1995. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. Morb. Mortal. Wkly. Rep. **44:**590–591.
12. **Cooke, W., and N. H. Bartenhagen.** 1994. Seroreactivity to Borrelia burgdorferi antigens in the absence of Lyme disease. J. Rheumatol. **21:**126–131.
13. **Coyle, P., S. E. Schutzer, A. L. Belman, L. B. Krupp, and M. G. Golightly.** 1990. Cerebrospinal fluid immune complexes in patients exposed to Borrelia burgdorferi: detection of Borrelia-specific and -nonspecific complexes. Ann. Neurol. **28:**739–744.
14. **Craven, R. B., T. J. Quan, R. E. Bailey, R. Dattwyler, R. W. Ryan, L. H. Sigal, A. C. Steere, B. Sullivan, B. J. Johnson, D. T. Dennis, and D. J. Gubler.** 1996. Improved serodiagnostic testing for Lyme disease: results of a multicenter serologic evaluation. Emerg. Infect. Dis. **2:**136–140.
15. **Digeon, M., M. Laver, J. Riza, and J. F. Bach.** 1977. Detection of circulating immune complexes in human sera by simplified assays with polyethylene glycol. J. Immunol. Methods. **16:**165–183.
16. **Dressler, F., J. A, Whalen, B. N. Reinhardt, and A. C. Steere.** 1993. Western blotting in the serodiagnosis of Lyme disease. J. Infect. Dis, **167:**392–400.
17. **Engstrom, S., E. Shoop, and R. C. Johnson.** 1995. Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. J. Clin. Microbiol. **33:**419–427.
18. **Fikrig, E., E. D. Huguenel, R. Berland, D. W. Rahn, J. A. Hardin, and R. A. Flavell.** 1992. Serologic diagnosis of Lyme disease using recombinant outer surface proteins A and B and flagellin. J. Infect. Dis. **165:**1127–1132.
19. **Fleiss, J.** 1981. Statistical methods for rates and proportions, 2nd ed., p. 328. John Wiley & Sons, New York, N.Y.
20. **Grodzicki, R., and A. C. Steere.** 1988. Comparison of immunoblotting and indirect enzyme-linked immunosorbent assay using different antigen preparations for diagnosing early Lyme disease. J. Infect. Dis. **157:**790–797.
21. **Guner, E. S.** 1994. Retention of B. burgdorferi pathogenicity and infectivity after multiple passages in a co-culture system. Experientia **50:**54–59.
22. **Hansen, K., P. Hindersson, and N. S. Pedersen.** 1988. Measurement of antibodies to the *Borrelia burgdorferi* flagellum improves serodiagnosis in Lyme disease. J. Clin. Microbiol. **26:**338–346.
23. **Hansen, K., and A. M. Lebech.** 1993. Lyme neuroborreliosis: a new sensitive diagnostic assay for intrathecal synthesis of Borrelia burgdorferi specific immunoglobulin G, A, and M. Ann. Neurol. **30:**197–205.
24. **Hardin, J., A. C. Steere, and S. E. Malawista.** 1979. Immune complexes and the evolution of Lyme arthritis. Dissemination and localization of abnormal C1q binding activity. N. Eng. J. Med. **301:**1358–1363.
25. **Hardin, J., L. C. Walker, A. C. Steere, T. C. Trumble, K. S. K. Tung, R. C. Williams, Jr., S. Ruddy, and S. E. Malawista.** 1979. Circulating immune complexes in Lyme disease. Detection by ^{125}I-C1. binding C1, solid phase, and Raji cell assays. J. Clin. Investig. **63:**468–477.
26. **Hedberg, C., M. T. Osterholm, K. L. MacDonald, and K. E. White.** 1987. An interlaboratory study of antibody to Borrelia burgdorferi. J. Infect. Dis. **155:**1325–1327.
27. **Hilton, E., A. Tramontano, J. DeVoti, and S. K. Sood.** 1997. Temporal study of immunoglobulin M seroreactivity to *Borrelia burgdorferi* in patients treated for Lyme borreliosis. J. Clin. Microbiol. **35:**774–776.
28. **Jiang, W., B. J. Luft, W. Schubach, R. J. Dattwyler, and P. D. Gorevic.** 1992. Mapping the major antigenic domains of the native flagellar antigen of *Borrelia burgdorferi*. J. Clin. Microbiol. **30:**1535–1540.
29. **Johnson, B., K. E. Robbins, R. E. Bailey, B.-L. Cao, SL. Sviat, R. B. Craven, L. W. Mayer, and D. T. Dennis.** 1996. Serodiagnosis of Lyme disease: accuracy of a two-step approach using a flagella-based ELISA and immunoblotting. J. Infect. Dis. **174:**346–353.
30. **Kaell, A., P. R. Redecha, K. B. Elkon, M. G. Golightly, P. R. Schulman, R. J. Dattwyler, D. L. Kaell, R. D. Inman, C. L. Christian, and D. J. Volkman.** 1993. Occurrence of antibodies to Borrelia burgdorferi in patients with nonspirochetal subacute bacterial endocarditis. Ann. Intern. Med. **119:**1079–1083.
31. **Kalish, R., J. M. Leong, and A. C. Steere.** 1995. Early and late antibody responses to full-length and truncated constructs of outer surface protein A of *Borrelia burgdorferi* in Lyme disease. Infect. Immun. **63:**2228–2235.
32. **Karlsson, M., and M. Granstrom.** 1989. An IgM-antibody capture enzyme immunoassay for serodiagnosis of Lyme borreliosis. Serodiagn. Immunother. Infect. Dis. **3:**413–421.
33. **Ley, C., C. Le, E. M. Olshen, and A. L. Reingold.** 1994. The use of serologic tests for Lyme disease in a prepaid health plan in California. JAMA **271:**460–463.
34. **Lovece, S., R. Stern, and L. J. Kagen.** 1991. Effects of rheumatoid factor, antinuclear antibodies and plasma reagin on the serologic assay for Lyme disease. J. Rheumatol. **18:**1813–1818.
35. **Luger, S., and E. Krauss.** 1990. Serologic tests for Lyme disease—interlaboratory variability. Arch. Intern. Med. **150:**761–763.
36. **Magnarelli, L., J. M. Meegan, J. F. Anderons, and W. A. Chappell.** 1984. Comparison of an indirect fluorescent-antibody test with an enzyme-linked immunosorbent assay for serological studies of Lyme disease. J. Clin. Microbiol. **20:**181–184.
37. **Magnarelli, L., J. G. F. Anderson, and R. C. Johnson.** 1987. Cross-reactivity in serological tests for Lyme disease and other spirochetal infections. J. Infect. Dis, **156:**183–188.
38. **Magnarelli, L., J. F. Anderson, and A. G. Barbour.** 1989. Enzyme-linked immunosorbent assays for Lyme disease: reactivity of subunits of Borrelia burgdorferi. J. Infect. Dis. **159:**43–49.
39. **Magnarelli, L. A., J. N. Miller, J. F. Anderson, and G. R. Riviere.** 1990. Cross-reactivity of nonspecific treponemal antibody in serologic tests for Lyme disease. J. Clin. Microbiol. **28:**1276–1279.
40. **Magnarelli, L. A., E. Fikrig, S. J. Padula, J. F. Anderson, and R. A. Flavell.** 1996. Use of recombinant antigens of *Borrelia burgdorferi* in serologic tests for diagnosis of Lyme borreliosis. J. Clin. Microbiol. **34:**237–240.
41. **Melski, J., K. D. Reed, P. D. Mitchell, and D. G. D. Barth.** 1993. Primary and secondary erythema migrans in central Wisconsin. Arch. Dermatol. **129:**709–716.
42. **Mitchell, P., K. D. Reed, M. F. Vandermause, and J. W. Melski.** 1993. Isolation of Borrelia burgdorferi from skin biopsy specimens of patients with erythema migrans. Am. J. Clin. Pathol. **99:**104–107.
43. **Mitchell, P., K. D. Reed, T. L. Aspeslet, M. F. Vandermause, and J. W. Melski.** 1994. Comparison of four immunoserologic assays for detection of antibodies to *Borrelia burgdorferi* in patients with culture-positive erythema migrans. J. Clin. Microbiol. **32:**1958–1962.
44. **Nachamkin, I., D. L. Riddle, M. Feldman, and P. H. Edelstein.** 1996. Utilization of tests for Lyme disease antibody at a university hospital. Clin. Diagn. Lab. Immunol. **3:**287–289.
45. **Nishio, M., M. R. Liebling, A. Rodrigues, L. H. Sigal, and J. S. Louie.** 1993. Identification of *Borrelia burgdorferi* using interrupted polymerase chain reaction. Arthritis Rheum. **36:**665–675.
46. **Pollack, R., S. R. Telford III, and A. Spielman.** 1993. Standardization of medium for culturing Lyme disease spirochetes. J. Clin. Microbiol. **31:**1251–1255.
47. **Rose, C., P. T. Fawcett, K. M. Gibney, and R. A. Doughty.** 1996. Residual serologic reactivity in children with resolved Lyme arthritis. J. Rheumatol. **23:**367–369.
48. **Schutzer, S., P. K. Coyle, A. L. Belman, M. G. Golightly, and J. Drulle.** 1990. Sequestration of antibody to Borrelia burgdorferi in immune complexes in seronegative Lyme disease. Lancet **335:**312–315.
49. **Schutzer, S., P. K. Coyle, J. J. Dunn, B. J. Luft, and M. Brunner.** 1994. Early and specific antibody response to ospA in Lyme disease. J. Clin. Investig. **94:**454–457.

49a. **Schutzer, S. E., P. K. Coyle, P. Reid, and B. Holland.** 1999. *Borrella burgdorferi*-specific immune complexes in acute Lyme disease. JAMA **282:**1942–1946.

50. **Schwartz, B., M. D. Goldstein, J. M. C. Ribeiro, T. L. Schulze, and S. I. Shahied.** 1989. Antibody testing in Lyme disease—a comparison of results in four laboratories. JAMA **244:**3431–3434.
51. **Seltzer, E., and E. D. Shapiro.** 1996. Misdiagnosis of Lyme disease: when not to order serologic tests. Pediatr. Infect. Dis. J. **15:**762–763.
52. **Siegel, S., and N. Castellan.** 1988. Nonparametric statistics for the behavioral sciences, 2nd ed., p. 75–80. McGraw-Hill, New York, N.Y.
53. **Sigal, L. H, A. C. Steere, D. H. Freeman and J. M. Dwyer.** 1986. Proliferative responses of mononuclear cells in Lyme disease: reactivity to *Borrelia burgdorferi* antigens is greater and the response to mitogens is less in joint fluid than in blood. Arthritis Rheum. **29:**761–769.
54. **Sigal, L. H.** 1994. Persisting complaints of Lyme disease: a conceptual review: Am. J. Med. **96:**365–374.
55. **Sigal, L. H.** 1996. The Lyme disease controversy: the social and financial

costs of the mismanagement of Lyme disease. Arch. Intern. Med. **156:**1493–1500.
56. **Steere, A. C.** 1989. Lyme disease. N. Eng. J. Med. **321:**586–596.
57. **Steere, A., V. P. Berardi, K. E. Weeks, E. L. Logigian, and R. Ackermann.** 1990. Evaluation of the intrathecal antibody response to Borrelia burgdorferi as a diagnostic test for Lyme neuroborreliosis. J. Infect. Dis. **161:**1203–1209.
58. **Steere, A., E. Taylor, G. L. McHugh, and E. L. Logigian.** 1993. The overdiagnosis of Lyme disease. JAMA **269:**1812–1816.
59. **von Stedingk, L.-V., M. von Stedingk, H.-G., Hardemark, A. Brauner, E. Kindstrand, A. Hovmark and E. Asbrink.** 1990. Intrathecally produced antibodies to Borrelia burgdorferi measured by IgG-capture ELISA. Serodiagn. Immunother. Infect. Dis. **4:**387–394.
60. **Weiss, N., V. A. Sadock, L. H. Sigal, M. R. Phillips, P. F. Merryman and S. B. Abramson** 1995. False positive seroreactivity to *Borrelia burgdorferi* in systemic lupus erythematosus: the value of immunoblot analysis. Lupus **4:**131–137.
61. **Yu, Z., J. M. Carter, H. Lackland, L. H. Sigal and S. Stein.** 1996. Presentation of peptide antigens as albumin conjugates for use in detection of serum antibodies by ELISA. Bioconj. Chem. **7:**338–342.
62. **Zoller, L., M. Haude, D. Hassler, S. Burkard, and H. G. Sonntage.** 1989. Spontaneous and post-treatment antibody kinetics in late Lyme borreliosis. Serodiagn. Immunother. Infect. Dis. **3:**345–353.